EXPLORING NEW USES OF EXISTING DRUGS AGAINST LIVER STAGE MALARIA

by
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Abstract

Malaria, as one of the most lethal infectious diseases, has been causing much morbidity and mortality worldwide for decades. The causative parasites, *Plasmodium* spp., are extremely hard to eliminate because of their intricate life cycle and fast-emerging drug resistance. At present most malaria drug development programs have focused on the blood stages for curative treatment of malaria disease. The liver stage of malaria, an obligatory step of disease progression prior to blood stages, is an area of drug development that has been omitted for a long time because it is cumbersome to grow hepatocytes and parasites for drug screening assays. Liver stage drugs are important for the prophylaxis of all malarias as well as replacing toxic primaquine for dormant stages of *P. vivax* and *P. ovale* malaria. Because of the scarcity and urgent need of drugs specific for liver stage malaria, the purpose of this study is to identify novel, potent and non-toxic therapeutic agents from existing drugs and compounds. In collaboration with Gradient Biomodeling we used a virtual screen with a quantum physics model to identify potential novel target molecules. Data from a handful of liver stage *in vitro* screens including the Novartis dataset of 5,000

molecules was input to create a pharmacophore model and generate similarities utilizing additional quantum physics properties. We screened *in silico* by quantum modeling many millions of molecules to identify 45 unique potential compounds based on quantum scores. We obtained or synthesized nearly a dozen of those with half showing activity both in single oral dose in mice and in an *in vitro* liver stage *P. berghei* model. Prominent amongst the list was an isomer of cethromycin, which is a 3 quinoline with a ketolide. This was active with more than a log decrease in mice and acted synergistically with primaquine. Another simple concept used was identification of drugs with enterohepatic circulation that reach high hepatocyte concentrations relative to plasma like azithromycin, posaconazole, moxifloxacin and even cethromycin. The macrolide hybrids to quinoline may be a potent nontoxic set of molecules for liver stage malaria.

Thesis readers

Dr. David Sullivan, Department of Molecular Microbiology and Immunology

Dr. Sean Prigge, Department of Molecular Microbiology and Immunology

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Introduction

Malaria

Malaria has been a lethal threat to people, especially children under 5 years old, in endemic areas of Africa, Asia and the Americas. According to the latest report, this parasitic disease has resulted in approximately 207 million cases and 627,000 deaths worldwide in 2012[1]. High malaria morbidity and mortality have caused a catastrophic impact on the local economic and social development[2,3]. The amount of investments and control programs has dramatically increased since 2000. To be more specific, the number of households in sub-Saharan Africa owning at least one insecticide-treated bed net (ITN), an important part of vector control, has multiplied almost 20 times from 2000 to 2012[1]. In addition to ITNs, indoors insecticide spraying plays a role in preventing mosquitoes from transmitting the disease to humans and rapid diagnostic testing is more readily available. In 2005 there were only about 200,000 RDT's distributed but in 2012 the number increased to more than 108 million. Likewise the number of artemisinin combination treatments has increased from 11 million in 2005 to 330 million in 2012. As a result, the mortality rate of

malaria has been reduced significantly by 42% in all ages and even higher among children under 5 years of age, which encouraged WHO to further set the goal towards 75% by 2015[1]. As for prevention, despite all the efforts towards a long-desired effective vaccine, the best possible candidates we have until now are made of irradiated or attenuated whole sporozoites[4–6]. Therefore, chemotherapy remains a vital part of weaponry in the battle against malaria[7].

Liver stage

One of the reasons that malaria is so hard to control and treat is its intricate life cycle between human and mosquito hosts. Moreover, the disease progression in the human consists of multiple stages and forms of the parasite. When an infected mosquito takes a blood meal, approximately one hundred sporozoites are injected and deposited under the host's skin. Later these sporozoites can find their way to peripheral blood vessels in which they can eventually travel to liver sinusoids[8]. Sporozoites will traverse through several hepatocytes in search of a habitable one before they form an intracellular parasitophorous

vacuole in which growth and replication take place[9]. The number of days that parasites need to progress to the next stage by releasing thousands of merozoites produced in hepatocytes usually varies from species to species. The free-living merozoites then go on to invade erythrocytes, where the blood stage begins and leads to massive multiplication of even more parasites[10]. Malaria remains asymptomatic in patients until this burst production of parasites at the blood stage that results in serious symptoms and disease[11]. Due to this strong association between disease onset and parasite localization, most antimalarial measures including drug development have been focused on blood stages, leaving the importance of liver stage neglected by malaria community for a long time. In fact, if we want to practically cure and eventually eradicate malaria, killing liver stage parasites has several advantages compared to targeting blood stage parasites[2,7]. Because only a few sporozoites can actually reach and grow in hepatocytes after a mosquito bite, the liver stage is considered as a bottleneck of parasite development and we can stop the disease progression by eliminating parasites in the liver with effective pharmaceutical agents. Theoretically, a person receiving treatment for malaria who was bitten by another mosquito

a few days before treatment could still have liver stages not treated by artemisinin combination therapy. These liver parasites could be released after 6-10 days when drug levels are falling in the blood. Ideal drugs should be active against liver, blood and gametocyte stages. Tafenoquine shows promise for action against all stages but is still awaiting regulatory approval.

Antimalarial drug development for liver stage

Chemotherapy has been the most powerful strategy against malaria ever since 200 BC when Chinese herbalists first used Qinghaosu, also known as artemisinin, to treat patients. Another ancient drug, quinine, was first used in the 1600's. Over the decades in 1900s, several new classes of antimalarial drugs had been discovered and widely used, including chloroquine, pyrimethamine, primaquine, atovaquone, the antibacterial apicoplast targeted drugs like azithromycin and so on. Among these drugs, primaquine (Table 3) is the only clinically approved one that has been shown capable of clearing both growing and dormant *Plasmodium* parasites in the liver[12]. Besides, most of the drug screening and discovery

projects have been concentrating on blood stages in the endeavor to treat the clinical symptoms[13], leaving us with the scarcity of drugs against liver stage malaria. What makes this situation even worse is that administration of primaquine is always accompanied by some severe side effects like nausea and hemolysis (in patients with G6PD deficiency)[14]. Atovaquone is another approved drug for malaria prevention. While atovaquone can kill off actively growing parasites in the liver, it has no effect on the dormant hypnozoites of *P. vivax* and *P. ovale*[15], which can lead to relapses of the disease long after clearance of parasites in the blood stream. It is evident that discovering new drugs against liver stage malaria is both necessary and urgent considering its critical role in the prevention of disease progression and relapse.

Selection of novel liver stage drug candidates

In order to overcome the scarcity of drugs against liver stage malaria, this study mainly focused on discovering new potential leads among existing drugs and compounds. Various strategies were applied to identify potential candidates from the literature and

databases based on their pharmacokinetic or quantum properties. Positive selections were further tested in both *in vitro* and *in vivo* assays to validate their inhibitory effect on parasite growth in hepatocytes.

Enterohepatic circulation: Posaconazole and Bedaquiline

About a liter of bile is made daily in the adult. Drug uptake into hepatocytes is by both carrier mediated transporters for polar drugs and by diffusion for lipophilic drugs. Cardiac glycosides, anion and cations have three separate active transporters. These actively secreted drugs are charged when entering the intestinal tract and are not resorbed. While molecular weight predominates in prediction of drugs with biliary route of elimination, other factors also influence the biliary route over the urinary route of drug elimination. In general, a molecular weight above 450 to 500 is favorable threshold for biliary excretion that progressively increases from about 15% for drugs below 450 MW to almost 100% for molecular weight above 600 to 700. In a study of similar cephalosporins[16] addition of a hydroxide to a phenolic group halved biliary excretion for a cephalosporin of 575 MW.

Many other drugs that enter the bile into the upper intestine are passively absorbed via the intestines into the blood and passed through the hepatocytes into the bile again. This process is called enterohepatic circulation. Conjugation by the cytochrome P450 system generally enhances biliary elimination. Chloramphenicol is conjugated to the glucuronide, passes into the bile then the glucuronide is cleaved in the intestines by bacteria and the drug is resorbed to repeat the process. In this fashion drugs can undergo a lengthy enterohepatic circulation[17–19].

If a drug favors enterohepatic circulation, it should be considered as therapeutically advantageous for liver stage malaria because recycling within the system can dramatically prolong the availability of administered drug to parasite-infected hepatocytes as well as increasing effective concentration in the liver. With this notion in mind, a search among scientific literatures was conducted and identified two existing drugs with potential liver stage antimalarial activity.

Posaconazole (Table 3) is mostly used as an antifungal agent to treat diseases induced by *Candida*[20] and *Aspergillus*[21] species. The drug is effective for both acute and chronic

Chagas disease[22]. In their recent study, a Brazilian research group showed posaconazole's antimalarial activity with a relatively low IC_{50} of 2.6 μM for blood stages and ability to reduce *P. berghei* parasitemia by 71% when given to infected mice orally[23]. Furthermore, posaconazole was proved by other researchers to be extensively distributed in tissues and mainly excreted by feces after oral administration[24–26]. These properties of posaconazole implied its potential for liver stage antimalarial activity.

Bedaquiline, the first new tuberculosis drug in more than forty years, was first discovered in 2005 by Koen Andries and his team[27]. According to this article, bedaquiline is a diarylquinoline compound with a substructure similar to quinoline and effective against multi-drug resistant *M. tuberculosis* species (Table 3). Bedaquiline selectively targets the *Mycobacterium tuberculosis* F_1F_0 ATP synthase, which in *Plasmodium* blood stages is present but not thought to be functional. More importantly, pharmacokinetic studies showed that orally administered bedaquiline was able to reach high concentration in tissues including liver and had an effective half life as long as 24 hours, which may overcome the lack of selectivity for the parasites. Additionally bedaquiline is a 1,2

substituted quinoline with a bromine in the 7 position. Taking these facts into consideration, bedaquiline would was a reasonable candidate drug for liver stage malaria testing.

In silico quantum modeling: Cethromycin and other compounds

Our collaborators in Gradient Biomodeling, LLC have developed and published an unconventional search technology for pharmaceutical agents, in which compounds are described rigorously based on their quantum properties rather than chemical structures. By inputting quantum descriptors of known antimalarial drugs as filters, the system can screen *in silico* through thousands of drugs and compounds to identify matches bearing similar quantum properties. Because these quantum attributes are usually associated with corresponding pharmacological activities, this method can serve as a powerful tool for discovering novel, potent and non-toxic antimalarial drugs that act in a similar manner as known drugs. Since this method has already been validated in screening compounds with blood stage antimalarial activity[28], a new modeling system was established referring to chemicals that are effective for liver stage malaria for identifying prospective drugs. A

battery of 5757 compounds was generated by combining Novartis ChEMBL-NTD dataset[29] and validated liver stage antimalarial drugs from recent publications that investigated *in vitro* hepatocyte *Plasmodium* inhibition. These compounds were utilized to establish the modeling system that later screened over 65 million compounds from various databases *in silico*, identifying 45 potential candidates.

The model matches the input molecules with identified candidates over numerous quantum scoring criteria. A representative image of matching the quantum components is shown in Figure 1 for A8 and GNF-Pf-1498 where the greater weight is given to the nonmacrolide quinoline on A8 and the nitrogen rich aromatic of GNF-Pf-1498 shown in red. A sample of matching quantum scores is shown in Table 1. Table 2 identifies the matched quantum score with the reference input molecules from the 5,000 compounds screened in the Meister data set. Cyclosporin A has a liver stage inhibition of 1.7 nM, monensin 0.001 nM and telithromycin 280 nM.

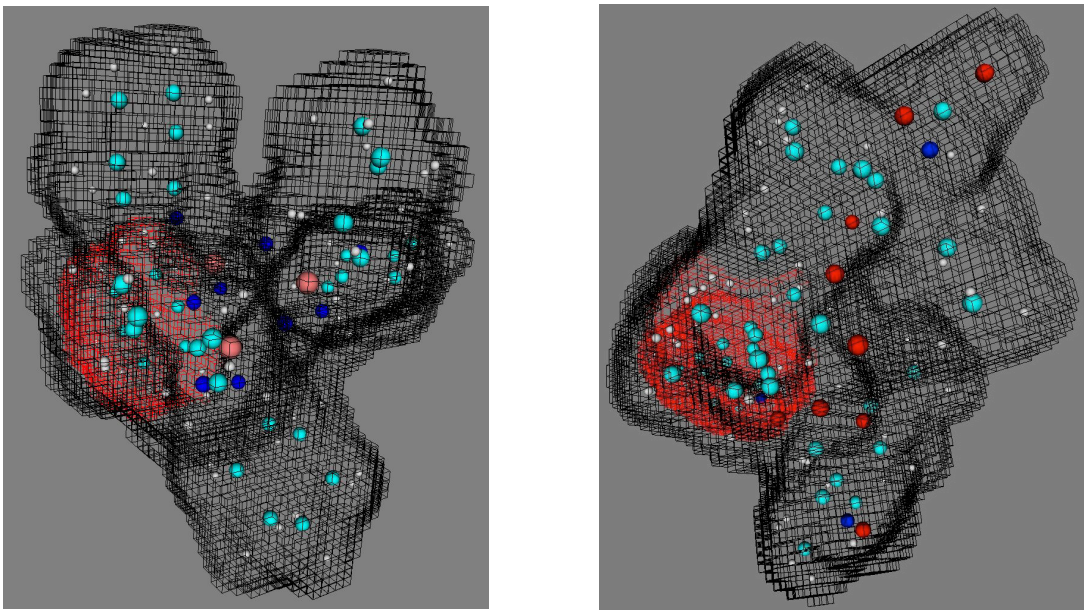
input	CyclosporinA	0	0	0	1(0;1)(1)	1(1;1)(1)	0	1(1;1)(3)	1(1;1)(4)	0	1(1;2)(1;2)
output	A3	1(1;1)(1)	1(1;1)(1)	1(0;1)(1)	1(0;1)(1)	1(0;1)(1)	0	1(1;1)(3)	1(0;5)(1;2;3;4;5)	0	1(0;2)(1;2)
output	A1	1(1;1)(1)	1(1;1)(1)	1(1;1)(1)	1(0;1)(1)	1;3)(1;2;	0	1(1;1)(3)	1(0;5)(1;2;3;4;5)	0	1(0;3)(1;2;4)

input	Monensin	0	0	0	1(0;1)(1)	1(1;1)(1)	0	1(1;1)(1)	1(1;1)(1)	0	1(1;2)(1;2)
input	Telithromycin	0	0	0	1(0;1)(1)	1(1;1)(1)	1(1;1)(1)	1(1;1)(1)	1(1;1)(1)	0	1(1;2)(1;2)
output	A8	0	0	0	1(0;1)(1)	1(1;1)(1)	1(1;1)(1)	1(1;1)(1)	1(1;1)(1)	0	1(1;2)(1;2)

Table 1. Matching quantum scores of input and output molecules for A1, A3 and A8. A1, A3 and A8 are matched with known anti-malarial agent cyclosporin A and monensin respectively based on similar quantum properties.

ID of novel molecule	Quantum pattern score	Reference molecule
A10	26	GNF-Pf-1498
A5	22	Monensin; Saquinavir; Sirolimus; Everolimus; GNF-Pf-1498
A6	22	GNF-Pf-1498; Monensin
A7	22	None
A3	21	Cyclosporin A
A14	20	None
A4	20	None
A12	20	Sirolimus; Everolimus
A1	19	Cyclosporin A
A15	19	Cyclosporin A; Everolimus
A9	18	Everolimus
A11	18	Everolimus
A8	15	GNF-Pf-1498; Monensin

Table 2. Summary of quantum scores with molecule identifications and reference molecules for scoring. Molecules predicted by quantum modeling system are sorted by quantum pattern scores. Compounds marked with green were available from chemical suppliers and tested in this study. Higher score implies greater possibility of bearing anti-malarial activity.



GNF-Pf-1498 A8

Figure 1. Quantum similarity of GNF-Pf-1498 and the quinoline-macrolide hybrid A8 that is related to cethromycin.

Cethromycin is chemically similar to one of the potential candidates A8, with only a minor difference in the orientation of the attached quinoline substructure and an extra carbon in the linker between the macrolide and 3-quinoline. Cethromycin was safe to use for community acquired pneumonia but was not superior to existing drugs and was denied approval by the FDA on basis of efficacy. In addition to its anti-bacterial activity, cethromycin reaches a higher concentration in tissues than plasma. Along with cethromycin, seven other candidate compounds that were available from chemical suppliers were also tested for effectiveness.

Materials and Methods

Drugs and compounds

Posaconazole was purchased from Schering-Plough (Kenilworth, NJ). Bedaquiline was purchased from Adooq Bioscience (Irvine, CA). Quinoline hydrochloride, Erythromycin ethyl succinate, D-mannitol hexabenzoate, (R,R)-(-)-N,N'-Bis(3,5-di-tert-butylsalicylidene)-1,2-cyclohexanediaminomanganese(III) chloride and Hexa(o-tolyl)ditin were purchased from Sigma (St. Louis, MO). Amb356416, Amb3476561, Amb461470 and Amb1639503 were purchased from Ambinter (Orléans, France). Cethromycin was synthesized by and courtesy of Bryan T. Mott (NCATS, Bethesda, MD).

P. berghei sporozoites

P. berghei ANKA infected female *Anopheles stephensi* mosquitoes were dissected to obtain salivary glands. The glands were placed on ice in tubes containing Dulbecco's Modified Eagle's Medium (DMEM) and antibiotics. A homogenizer was used to gently

grind the glands and release the sporozoites. The yield of sporozoites was counted on a hemacytometer. After extraction, sporozoites were diluted in culture media and used for infecting hepatoma cells and mice.

In vitro assay

HepG2 cell culture

A circular coverslip was sterilized and placed in each well on a 24-well plate. HepG2, human hepatoma cells, were grown on these coverslips using Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 1X Penicillin-Streptomycin (Pen-Strep) at 37°C and 5% CO_2. 200,000 HepG2 cells were seeded in each well 24 hrs prior to infection.

Hepa1-6 cell culture

In each chamber of 8-well LabTek tissue culture slides, 50,000 mouse hepatoma cells, Hepa1-6, were seeded one day before infection. Cells were normally cultured in DMEM

supplemented with 10% FBS, 1X L-glutamine and 1X Pen-Strep at 37°C and 5% CO_2. Once incubated with *P. berghei* sporozoites, the culture medium supplement was changed to 2.5% FBS, 1X L-glutamine and 2X Pen-Strep.

Treatment preparation

Different drugs and compounds were dissolved based on individual solubility and stored at -20°C. Stock concentrations ranged from 1 mM to 32 mM. Before compounds were added to *P. berghei* infected cells, they were diluted in culture media to the desired concentrations.

Cytotoxicity assay

On a 96-well plate, 2500 cells in 250 μL culture medium were added in each well and incubated at 37°C and 5% CO_2 for 3 days so that the cells could reach log phase. Several wells were filled with only medium to serve as blank controls. 3 days later, spent medium was removed and fresh medium containing different dilutions of drugs was added to

designated wells. Each dilution was tested in triplicates. Then the plate was covered and returned to incubator. After 2 days, 25 µL of alamarBlue (Invitrogen) indicator was added to each well and the plate was further incubated for 3 hours. The plate was read spectrofluorometrically (excitation, 550nm; emission, 590nm) and percentage of cell growth in each drug dilution was calculated with the following formula: ((FI 590 of test agent dilution- FI 590 of blank)/(FI 590 of untreated control- FI 590 of blank))*100.

Infection of hepatocytes with Pb spz and Immunofluorescence assay

After removing spent medium, 200 µL of fresh medium containing 60,000 sporozoites extracted from infected mosquitoes were added to each chamber of hepatoma cell culture. Then the plate was spun down for 3 minutes at 300 x g and incubated at 37°C and 5% CO_2 for 3 hours after which sporozoite containing medium was aspirated off from each chamber. Before treatment with drugs, the cells were washed four times with DMEM containing 10X Pen-Strep and 5 ug/mL fungicide. Different treatments were added to infected cells and the plate was returned to incubator. After 24 hours, fresh culture medium

containing drugs was added to each chamber. Approximately 42 hours post-infection, the growth medium was removed and 100% cold methanol was added for 15 minutes to fix the cells. After washing with sterile PBS, cells were incubated in PBS with 5% FBS for blocking. Then 2E6 anti-HSP70 antibody was diluted to 10 μg/mL in PBS with 5% FBS and added in a 200 μL volume to cells. One hour later, cells were washed with PBS and incubated with 10 μg/mL Alexafour 594 anti-mouse secondary antibody. In order to visualize hepatocytes, nuclear counterstain DAPI was incubated with cells for 5 minutes and then washed off. The plastic chambers and silicone gasket were removed and slide was fully dried before mounted and sealed with cover slides. When preparation was complete, slide was taken to the fluorescence microscope for examination. Representative areas were randomly selected from each chamber and the numbers of infected cells were counted for a similar number of microscopic fields. The level of inhibition was determined by comparing the average of each chamber containing treated cells with that of the controls.

In vivo assay

Treatment preparation

Compounds that are soluble in water were dissolved in PBS, while those that are not soluble were prepared individually based on their solubility into oral suspensions. Final concentrations of compound dilutions ranged from 5 mg/mL to 10 mg/mL. All compound dilutions were freshly prepared and warmed at 37°C before being given to mice.

Infection of mice with Pb sporozoites and drug delivery

Six weeks old C57BL/6 mice with weight of 20-22 g were divided into groups of three. Before infection, mice were warmed with a heat lamp to dilate their tail veins for injection. A restrainer was used to hold each mouse in position and 12,000 Pb sporozoites in a 200 μL volume were injected into mice through tail veins with syringes. Two hours after injection, different dilutions of drugs in 100-200 μL volumes were delivered to mice by gavage using a plastic feeding tube. A second dose of drugs was given 24 hours after the first one to the mice that were on a daily dosing regimen.

Liver harvest of infected mice

Approximately 6 and 40 hours post-infection, mice infected with Pb sporozoites were anesthetized by inhaling Metofane® and sacrificed for harvesting whole livers. Each mouse liver was immediately put into 10 mL of Trizol® Reagent and fully homogenized. Then the homogenate was divided into small aliquots of 1 mL in Eppendorf tubes and stored at -80°C.

Whole tissue RNA isolation

Mouse liver homogenate in Eppendorf tubes was thawed on ice and resuspended by vortexing. Then 200 µL of chloroform (1/5 of initial volume) was added to each tube and mixed by inverting the tubes. After incubating for 3 minutes at room temperature, tubes were centrifuged at 12,000 x g and 4°C for 15 minutes. Centrifugation separates the mixture into three phases and RNA is exclusively contained in the top aqueous phase, which is usually yellowish due to high content of blood and iron in the liver homogenate.

The RNA containing aqueous phase was transferred to a new tube carefully without drawing any of the interphase that contains DNA. 500 µL of 100% isopropanol was added and tubes were inverted several times to precipitate RNA before further incubation for 10 minutes at room temperature. Eppendorf tubes were then centrifuged at 12,000 x g and 4°C for 10 minutes after which supernatant was removed without touching the RNA pellets. To wash RNA, 1 mL of 75% ethanol was added to resuspend pellets and tubes were centrifuged again at 7500 x g and 4°C for 5 minutes. Then 50-70 µL of ultra pure water heated to 55°C was added to each tube to dissolve RNA pellet when ethanol was completely removed. RNA dissolved in water was either kept on ice for upcoming experiment or stored at -80°C.

Real-time quantitative PCR

Isolated whole liver RNA was first used to generate cDNA by reverse-transcription PCR. The initial concentration of each RNA sample was measured on a NanoDrop microvolume spectrophotometer after which all samples were diluted to approximately

100 ng/mL for reverse-transcription reactions. For a 30 µL reaction, the components were set up as: 3.5 µL of nuclease-free water, 3 µL of 10X Buffer II and 10 mM dNTPs, 6 µL of $MgCl_2$ solution, 1.5 µL of 50 µM Random Hexamers, RNase Inhibitor and MuLV Reverse Transcriptase, 10 µL of RNA sample. cDNA products were stored at -20°C.

For a 10 µL real-time quantitative PCR, the components were set up as: 0.2 µL of 10 µM forward and reverse primer 18s *P. berghei*, 5 µL of 2X SYBR Green PCR Master Mix, 1.6 µL of nuclease-free water, and 3 µL of cDNA sample. The annealing temperature of the reaction was determined based on the predicted melting temperatures of the primers. To quantify the relative amounts of parasite specific protein and the murine Hypoxanthine Guanine Phosphoriboayltransferase (HPRT) housekeeping protein, a standard line was created using either plasmids with the coding gene or mixtures of randomly selected samples of designated concentrations. All test subjects were done in duplicates including positive and negative controls. When real-time PCR was finished, all data were baselined and normalized to the housekeeping gene before further analysis. The specific sequences of primers (The Core DNA Analysis Facility – JHU, Baltimore, MD) were: 5'-

GGAGATTGGTTTTGACGTTTATGCG-3’ and 5’-

AAGCATTAAATAAAGCGAATACATCCTTA-3’ for Pb ANKA 18s and 5’-

TCCCAGCGTCGTGATTAGC-3’ and 5’- CGGCATAATGATTAGGTATACAAAACA-3’

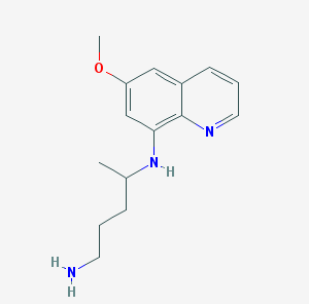
for mouse HPRT.

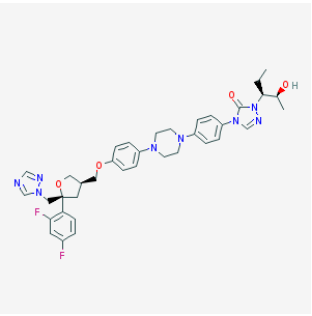
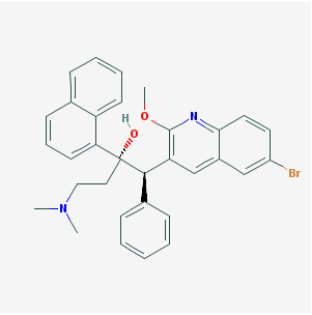
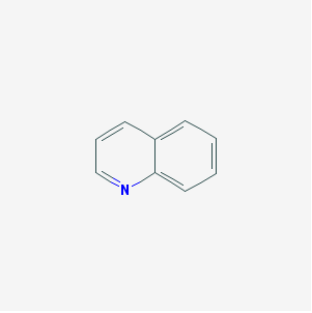
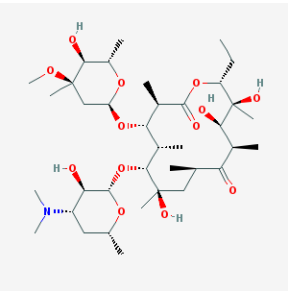
Results

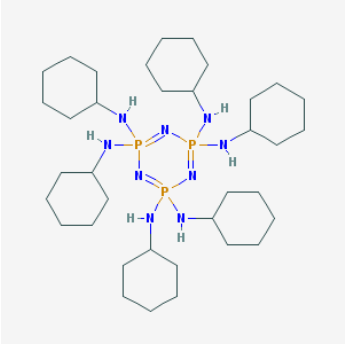
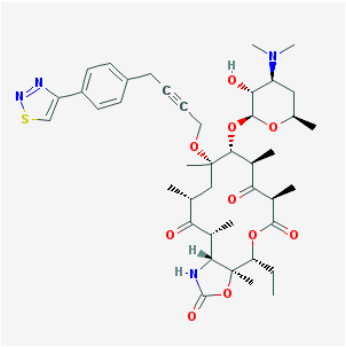
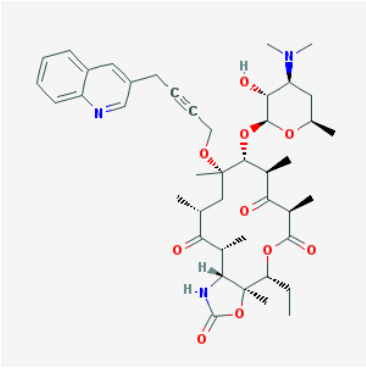
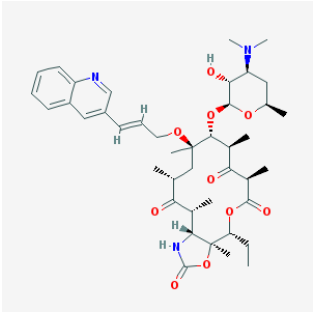
Nomenclature, abbreviation and chemical structure

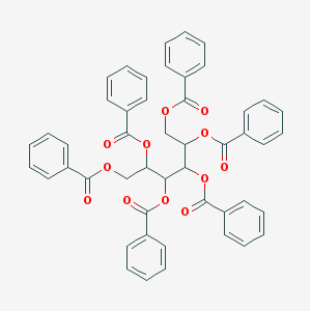
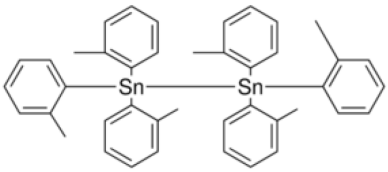
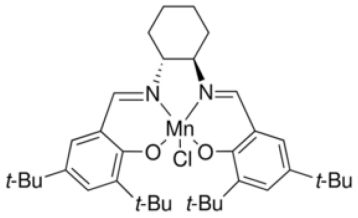
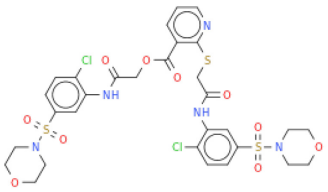
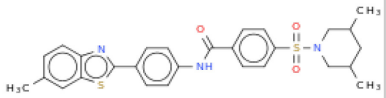
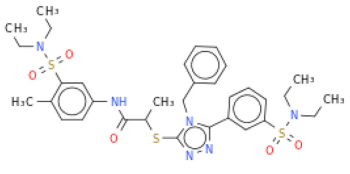
For unknown compounds, simplified names were assigned to each one for convenience. All drugs are also referred by their abbreviations in tables and graphs. In Table 3, all drugs and compounds are listed along with their individual molecular weights and chemical structures.

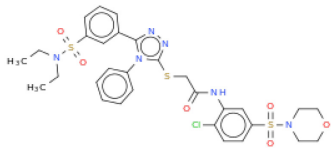
Table 3. List of drugs and compounds. Abbreviations and chemical structures of all tested drugs and compounds are shown in this table. Additional information related to chemical properties is listed. Compounds with molecular weights above 450 to 500 are favorable for enterohepatic circulation. XLogP3-AA is used to predict the lipophilicity of chemical, which increase with the value.

Drug/compound		Abbreviation	Chemical structure
Primaquine MW 259.3 XLogP3-AA H-Bond Donor H-Bond Acceptor Polar Surface Area	 2.2 2 4 60.2	PQ	

Posaconazole MW 700.8 XLogP3-AA 4.6 H-Bond Donor 1 H-Bond Acceptor 11 Polar Surface Area 112	PSZ	
Bedaquiline MW 555.5 XLogP3-AA 7.2 H-Bond Donor 1 H-Bond Acceptor 4 Polar Surface Area 45.6	BDQ	
Quinoline MW 129.2 XLogP3-AA 2 H-Bond Donor 0 H-Bond Acceptor 1 Polar Surface Area 12.9	QN	
Erythromycin MW 733.9 XLogP3-AA 2.7 H-Bond Donor 5 H-Bond Acceptor 14 Polar Surface Area 194	ERY	

GNF-Pf-1498 MW 723.9 XLogP3-AA 10 H-Bond Donor 6 H-Bond Acceptor 9 Polar Surface Area 109	GNF-Pf-1498	
CHEMBL300306 MW 811.0 XLogP3-AA 5.3 H-Bond Donor 2 H-Bond Acceptor 14 Polar Surface Area 204	A10	
CHEMBL440116 MW 777.9 XLogP3-AA 5.4 H-Bond Donor 2 H-Bond Acceptor 12 Polar Surface Area 163 (4 carbons in linker to inverted quinoline)	A8	
Cethromycin MW 765.9 XLogP3-AA 5.4 H-Bond Donor 2 H-Bond Acceptor 12 Polar Surface Area 163 (3 carbons in linker to quinoline)	CEY	

D-mannitol hexabenzoate MW 806.8 XLogP3-AA 9.8 H-Bond Donor 0 H-Bond Acceptor 12 Polar Surface Area 158	A9	
Hexa(o-tolyl)ditin MW 784.2	A11	
(R,R)-(-)-N,N'-Bis(3,5-di-tert-butylsalicylidene)-1,2-cyclohexanediaminomanganese(III) chloride MW 635.2	A7	
Amb356416 MW 788.7	A1	
Amb3476561 MW 519.6	A2	
Amb461470 MW 698.9	A3	

Amb1639503 MW 705.3	A4	

In vitro assay

Infection of hepatocytes with Pb spz and Immunofluorescence assay

All prepared slides were left overnight at 4°C after mounting and later examined under a fluorescence microscope. Because there were a great number of infected cells in most chambers, it was not practical to count the complete slide area manually. Therefore, 2-8 representative areas, varied in different experiments, were randomly selected for counting and calculating percentage of inhibition. The number of infected hepatoma cells treated with different drug and compound dilutions and percentages of inhibition are summarized in Table 4. In general, primaquine, posaconazole and bedaquiline show dose-dependent inhibitory effect on *in vitro* liver stage parasite infection. Among all drugs and compounds tested, primaquine at 20 μM and A3 at 10 μM are able to reduce parasite multiplication by over 90 percent, while bedaquiline at 40 μM, cethromycin at 20 μM and

A2 at 10 μM display relatively lower, but still promising inhibitory effect. Cethromycin at 40 μM and S3 at 10 μM were not countable because most hepatoma cells in these two chambers were killed by drugs' toxic effect.

In Figure 2a, large amount of untreated cells are infected and evenly distributed across the entire representative area, showing high rate of infection. In sharp contrast, only one cell in Figure 2b (PQ, 20 μM) and Figure 2c (A3, 10 μM) is infected. By comparing Figure 2d (BDQ 20 μM) and 2e (BDQ 40 μM), it is evident that there are fewer cells infected when treated with higher concentration of bedaquiline, suggesting a dose-dependent effect. Figure 2f (CEY, 40 μM) shows a representative area in which cell density is much lower than the others, indicating cytotoxicity induced by the high concentration of cethromycin.

	Treatment (μM)	Pb infected cells/area Mean±SEM	Inhibition of parasite growth (%)
Exp 1	PQ 10	13.17±0.73	61.9
	PQ 20	3.13±0.46	90.9
	PSZ 10	26.45±1.15	23.6
	PSZ 20	21.5±0.9	37.9
Exp 2	QN 20	122±10	11.6
	ERY 20	106±8	23.2
	QN 20+ ERY 20	97±4	29.7
Exp 3	BDQ 20	34.0±4.0	41.9
	BDQ 40	16.0±1.0	72.7
	CEY 20	28.0±3.0	52.1
	CEY 40	N/A	N/A
	A9 10	45.5±3.5	7.1
	A11 10	48.5±2.5	1.0
	A7 10	N/A	N/A
	A1 10	35.0±2.0	28.6
	A2 10	47.0±4.0	4.1
	A3 10	1.5±0.5	96.9
	A4 10	22.5±0.5	54.1

Table 4. Percent inhibition of infected hepatocytes treated with different drugs and compounds. Immunostained slides were examined under fluorescence microscope. The infection rate of hepatoma cells varied from experiment to experiment. Number of infected cells was averaged among randomly selected areas in each chamber and percentage of inhibition was determined relative to untreated cells.

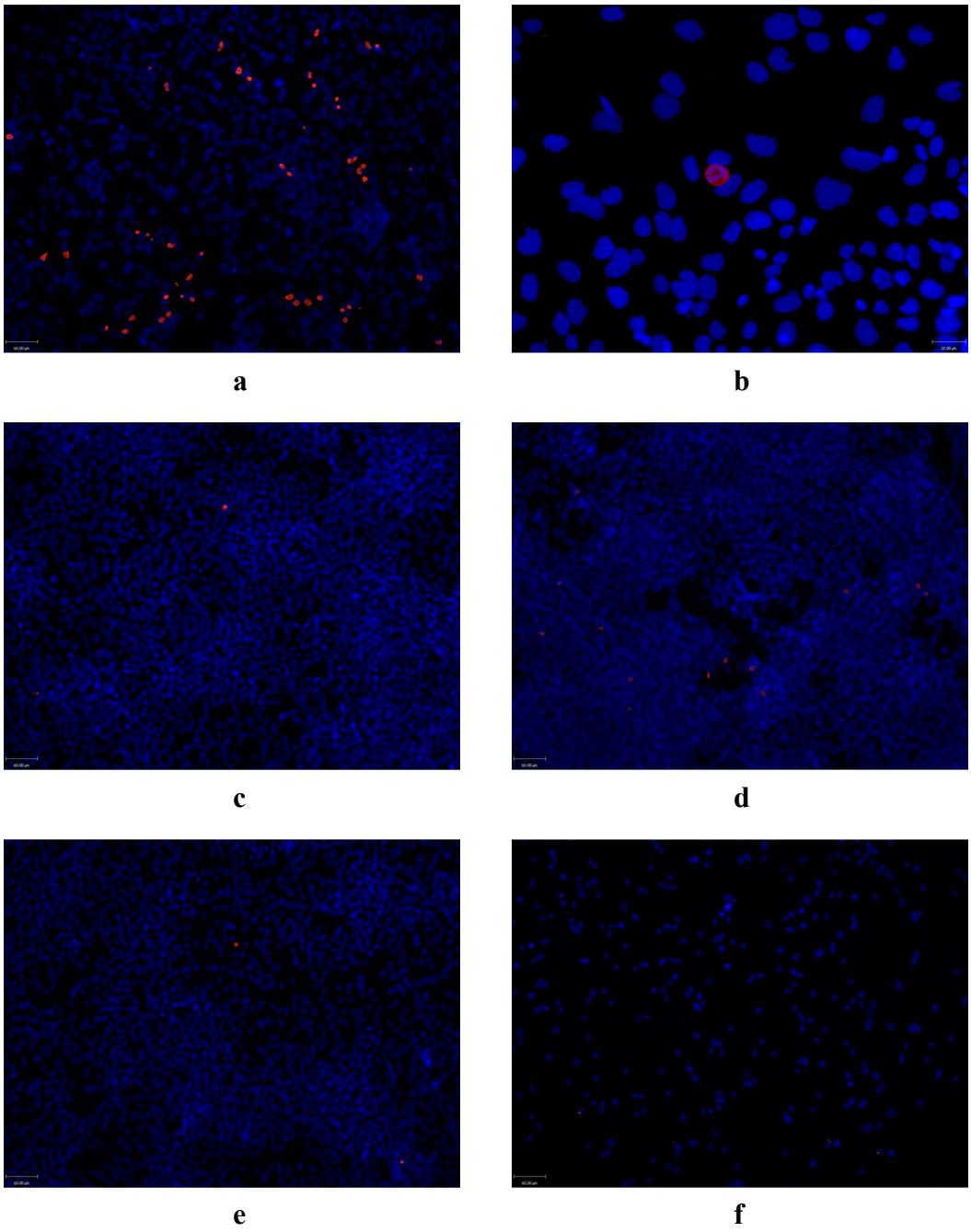


Figure 2. Immunofluorescence images of Pb infected hepatocytes treated with drugs and compounds. (a) Negative control: high infection rate of untreated cells; (b) PQ 20 µM and (c) A3 10 µM: parasite proliferation remarkably reduced; (d) BDQ 20 µM and (e) BDQ 40 µM: higher concentration of BDQ demonstrated greater inhibitory effect; (f) CEY 40 µM: decreased cell density induced by cytotoxicity. Blue: nucleus of hepatoma cells; Red: Pb liver stage parasites.

Cytotoxicity assay

Each drug or compound was tested in 5 different concentrations ranging from 5 μM to 80 μM and each concentration was done in triplicates. Fluorescence readouts were gathered and processed. The toxicity level of each drug or compound dilution was described as percentage growth of untreated cells. Figure 3 summarizes the cytotoxicity effects of all compounds and concentrations on hepatoma cells. Among approved drugs, both posaconazole and bedaquiline are well tolerated by cells in all tested concentrations but cethromycin and primaquine start to display toxicity when the concentration increases. However, the toxicity of cethromycin should be considered much less problematic than primaquine because at concentration of 40 μM more than 50 percent of cells can still survive in cethromycin while almost all cells are dead with 40 μM primaquine. In terms of the other compounds, there is a conspicuous and universal trend in which cytotoxicity increases substantially with concentration. As the most toxic test molecule of all, S3 remains lethal to hepatoma cells even at the lowest concentration of 5 μM.

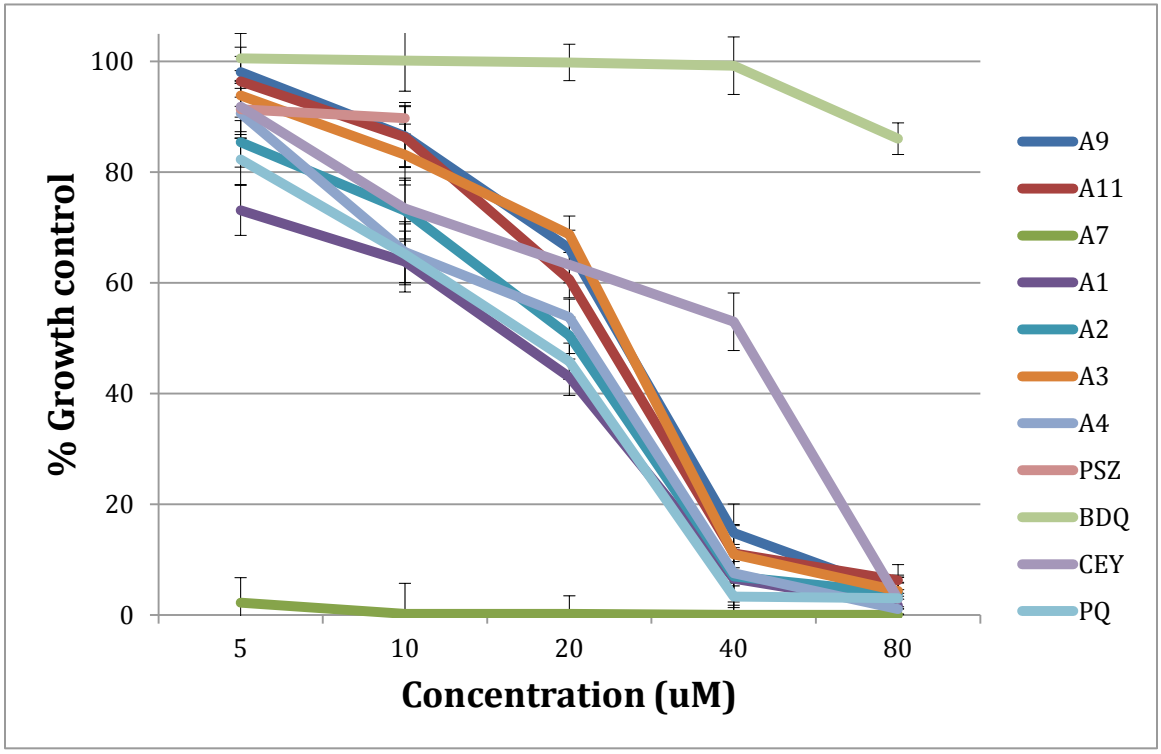


Figure 3. Cytotoxicity of drugs and compounds at different concentration, as measured by alamarBlue® indicator. Cells were seeded and incubated for 3 days to reach log phase and then treated with drugs and compounds for 2 days. Number of viable cells is positively correlated with strength of fluorescence. Background signal was deducted by measuring fluorescence of medium alone. Approved drugs including PSZ, BDQ and CEY showed mild to none toxicity to cells, while the other compounds gradually displayed toxicity with increasing concentration.

In vivo assay

According to a previous study, dosing mice infected by *P. berghei* with primaquine at 30 mg/kg can clear liver stage parasite. In this study, two different doses of primaquine were given to infected mice: 50 mg/kg to completely eliminate parasites in liver (positive

control) and 15 mg/kg to kill most of parasites while leaving a small portion for evaluating drug synergy.

Posaconazole and bedaquiline

Both drugs were tested in two different doses of 50 and 100 mg/kg in mouse model. Furthermore, bedaquiline at 100 mg/kg was combined with primaquine at 15 mg/kg to explore the possibility of synergistic effect between these two drugs. Due to bedaquiline's long half life, mice were given only one dose of the drug while the other drugs were administered twice, 24 hours apart from each other, before mice were sacrificed. As Figure 4 shows, both posaconazole and bedaquiline can inhibit parasite growth in mouse liver and their antimalarial activity is dose-dependent. Between these two drugs, bedaquiline is more potent, demonstrated by up to 70 percent reduction at 100 mg/kg. An even more important conclusion is that bedaquiline, when combined with a lower dose of primaquine, can reduce parasite load in liver to the same level as primaquine at 50 mg/kg, which is considered as elimination of parasites in this study.

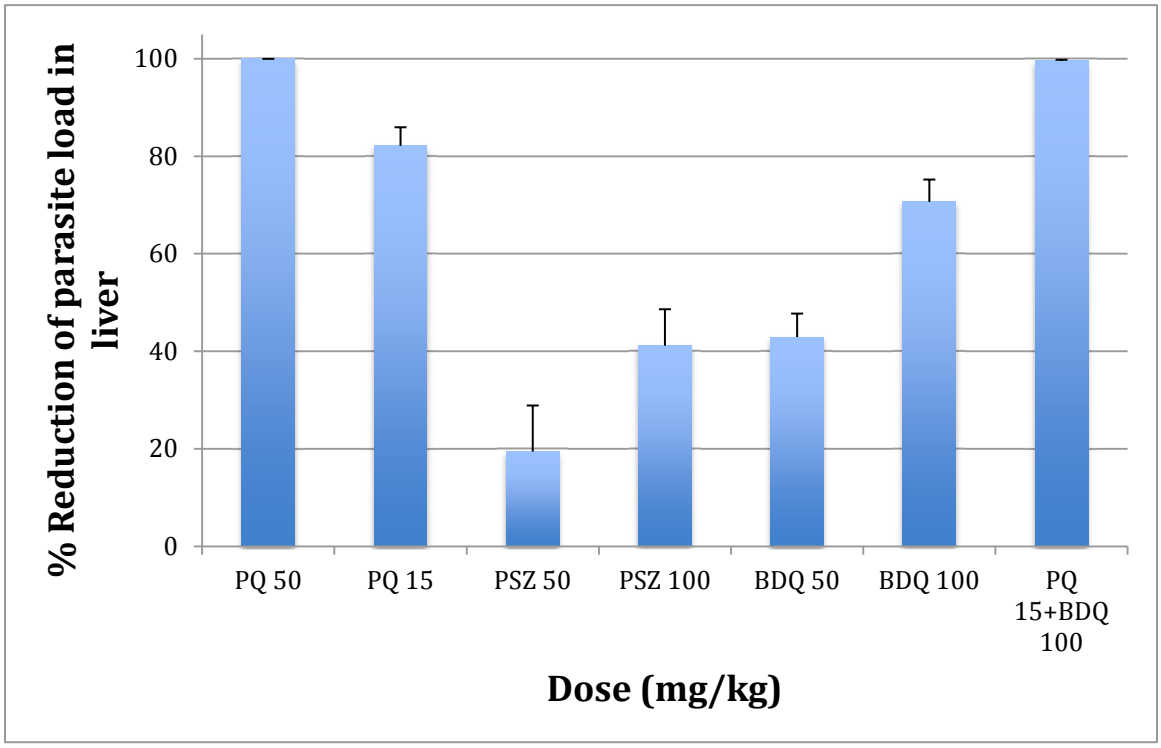


Figure 4. Real-time PCR quantification of parasite load in mice treated with BDQ and PSZ. BDQ was administered only once while the other drugs were given twice 24 hours apart from each dose. Both PSZ and BDQ demonstrated dose-dependent inhibitory effect while BDQ possessed higher potency. BDQ combined with low dose of PQ was able to clear actively growing parasites in mice liver.

Cethromycin and other compounds

In addition to cethromycin, erythromycin and quinoline were included in this experiment (Figure 5). Cethromycin and erythromycin share a remarkable similarity because the ketolide structure of cethromycin is derived from erythromycin by substituting the cladinose sugar with a keto-group and attaching a cyclic carbamate group in the lactone

ring[30]. Quinoline itself and combination with erythromycin were also tested to investigate if quinoline plays a role in the antimalarial activity of cethromycin. As in the previous experiment, cethromycin was tested in different doses and administered along with a lower dose of primaquine to explore synergistic drug effect. Of the seven compounds tested *in vitro*, only A1, A3 and A4 demonstrated measurable inhibitory effect on parasite infection of hepatoma cells. Therefore, these three compounds were further validated in mouse model at dose of 50 mg/kg. Due to limited availability of cethromycin, only one dose was given to mice while other drugs and compounds were administered twice.

Consistent with results from *in vitro* assay, quinoline, erythromycin and their combination measured only marginal inhibitory effect on parasite growth. Although cethromycin at 12 mg/kg only demonstrate minor effectiveness, a gradual dose response was observed after increasing the dose to 50 mg/kg, at which cethromycin was able to reduce parasite load by 60 percent. Again we were able to achieve parasite elimination by combining cethromycin at a relatively low dose of 12 mg/kg with primaquine at 15 mg/kg. While cethromycin at 12 mg/kg showed minimal inhibition at about 16%, combination

with low dose primaquine at 15 mg/kg (83% inhibition) showed higher 99% inhibition. Based on these results, it is safe to say that cethromycin is the most potent one of the tested drugs in this study. Although A3 compound did not demonstrate the same level of effectiveness as *in vitro* assay, all three drugs obtained from Ambinter (A1, A3 and A4) reduced parasite load by more than 50 percent without causing any serious or conspicuous side effect in mice after two doses.

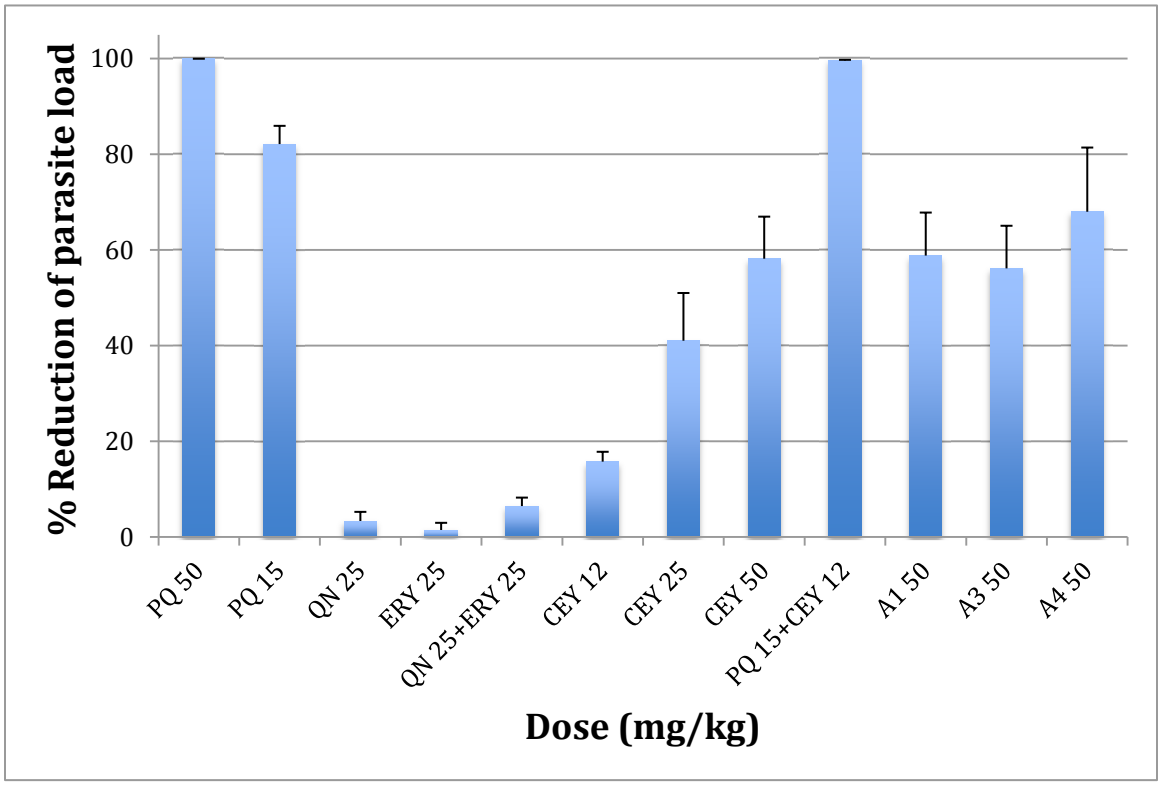


Figure 5. Real-time PCR quantification of parasite load in mice treated with CEY and other compounds**.** CEY was administered only once while the other drugs were given twice 24 hours apart from each dose. Two drugs related to CEY, QN and ERY, had only marginal effect on parasite growth. CEY's effectiveness increased with dosage, reaching 60% reduction at 50 mg/kg. CEY was also able to eliminate parasite infection when combined with low dose of PQ. All three compounds from Ambinter demonstrated significant inhibitory effect on parasite proliferation.

	Treatment (mg/kg)	Inhibition of parasite growth (%)	
		Mean	SEM
	PQ 50	99.9	0.0
	PQ 15	82.2	3.8
Cat 1	PSZ 50	19.5	9.4
	PSZ 100	41.2	7.5
	BDQ 50	42.9	4.8
	BDQ 100	70.7	4.6
	PQ 15+BDQ 100	99.7	0.2
Cat 2	QN 25	3.4	1.9
	ERY 25	1.5	1.5
	QN 25+ERY 25	6.5	1.8
	CEY 12	15.8	2.1
	CEY 25	41.1	10.0
	CEY 50	58.2	8.8
	PQ 15+CEY 12	99.7	0.1
Cat 3	A1 50	58.8	9.0
	A3 50	56.2	8.9
	A4 50	68.0	13.4

Table 5. Percent reduction of parasite load in livers of mice infected with *P. berghei* sporozoites. Mice were infected with *P. berghei* by intravenous injection of 12,000 sporozoites. All drugs and compounds except for CEY and BDQ were given twice every 24 hours. Parasite load in liver was determined by quantification of parasite specific 18s RNA with real-time PCR. Percentages of inhibition were calculated relative to respective untreated negative controls in different experiments.

Discussion

In order to eventually eradicate malaria, transmission prevention and curative treatment are two requirements that must be satisfied. However, this goal can not be achieved by targeting the blood stage for 4 reasons: 1) *Plasmodium* parasites undergo extensive multiplication in erythrocytes and spread out with blood flow, which makes them hard to clear from the body; 2) Massive replication dramatically increases the possibility of emergence of drug-resistant strains; 3) Unless the drug works on gametocytes as well, parasites can still be transmitted to mosquitoes when taking blood meals from infected humans; 4) *P. vivax and P. ovale* can form hypnozoites in hepatocytes and lead to relapses when they are reactivated at a much later date. In contrast, the liver stage provides us with a great opportunity to overcome these obstacles. Because there are many fewer multiplications in invaded hepatocytes, it is easier to achieve complete clearance of parasites including hypnozoites and the chance of drug resistance emergence is greatly limited. Once parasites are constrained and killed at the liver stage, disease no longer has the chance to progress to the blood stage, hence blocking any further infection or

transmission. Taking all advantages into consideration, the importance of the liver stage has surely been underestimated. Besides, the best option we have now for treating liver stage malaria is primaquine, which is not safe enough to be used widely. Under these circumstances, discovering novel and potent drugs for liver stage malaria becomes necessary and urgent and developing new drugs from the ground up in the conventional way is no longer applicable.

The ultimate purpose of this study is to discover liver stage antimalarial activity in existing drugs. Identification of potential candidate drugs was accomplished by searching for drugs that favor enterohepatic circulation or share quantum properties with known antimalarial drugs. To validate the effectiveness of each drug or compound, they were tested in both *in vitro* and *in vivo* assays. The *in vitro* assay, which is medium-throughput and manageable, was a modification of methods described before[31]. As for the *in vivo* test, we introduced a complete methodology that can quantify parasite load in whole liver in Pb infected mice with real-time fluorescence PCR. This test is advantageous because it not only mimics the actual infection and drug dynamics but also demonstrates effectiveness of

drugs more precisely.

Of the two drugs found to be extensively distributed in liver, posaconazole was previously proved to have blood stage antimalarial activity while bedaquiline carries a substructure similar to quinoline. Both drugs displayed dose-dependent effectiveness and are non-toxic to hepatoma cells. Although bedaquiline alone was not able to inhibit parasite growth completely, combination with low dose of primaquine reached same level of inhibition as primaquine at 50 mg/kg. This drug combination can be considered as a promising alternative to primaquine alone, a curative treatment with less toxic effect of primaquine.

Cethromycin and the other three Ambinter compounds demonstrated liver stage antimalarial activity as well. Cethromycin is closely related to compound A8 that shares quantum properties with GNF-Pf-1498 (Figure 1). Despite its minor cytotoxicity, cethromycin at a low dose of 25 mg/kg can reduce parasite load in mouse liver by approximately same percentage as bedaquiline at a dose two times higher, indicating cethromycin has a relatively higher potency. The dose of 25 mg/kg in the mouse is close to

the 150 mg single daily oral dose in adults, which is 3 mg/kg in humans. Mouse dosing conversion from humans multiplies by 11 or 12. The higher tolerated dose in humans is 300 mg/kg, which would be related to the 50 mg/kg dose in mice. Furthermore, cethromycin, when combined with low dose of primaquine, can also achieve parasite clearance in hepatocytes at a much lower dose than bedaquiline. The fact that the level of inhibition of cethromycin and primaquine combination was higher than the sum of their individual percentages implied a possible synergistic drug effect between these two drugs. The liver stage assay used here injects supraphysiologic doses of 10,000 sporozoites, which is almost 100 times the infectious dose of one hundred sporozoites that mosquitoes deliver. Cethromycin may be more effective at the more physiologic sporozoite inoculums. Further studies are warranted on cethromycin to measure blood and gametocyte stage inhibition concentrations. A1, A3 and A4 demonstrated effectiveness in both tests, further validating the feasibility of quantum modeling search technology. However, these three compounds are all toxic to hepatoma cells starting at 40 μM. To tackle this problem, the quantum components of these three compounds that are responsible for the antimalarial activity will

be located for molecular optimization or *de novo* drug design, during which cytotoxicity is reduced or removed while antimalarial activity is maintained.

Herein is reported a methodology of drug discovery and identification of three existing drugs and three compounds with liver stage antimalarial activity. Although further improvements are needed to generate more accurate results and increase throughput capacity, this is a practical attempt to discover novel, potent and non-toxic drugs against liver stage malaria. With incremental interest in liver stage drug development, we should see more of this attempted in near future.

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